

An assessment of peripheral immunity in patients with sarcoidosis using measurements of serum vitamin D3, cytokines and soluble CD23

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SUMMARY

The aetiology of the peripheral anergy in sarcoidosis is unclear. To investigate this further we measured the serum levels of several factors important in different aspects of immune regulation to obtain a profile of those factors which promote and inhibit immune activation in sarcoidosis. Thirty-seven patients with sarcoidosis and 20 healthy controls of similar sex and age comprised the study group. Serum IL-10, interferon-gamma (IFN- γ), soluble CD23 (sCD23), IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1 β and tumour necrosis factor-alpha (TNF- α) were measured using in-house ELISAs. Vitamin D3 was measured using a radioreceptor assay. Serum levels of sCD23 and IL-10 were significantly elevated in patients with sarcoidosis relative to controls (median 13.9 *versus* 9.5 arbitrary units/ml, $P < 0.01$ for sCD23, and 9.6 *versus* 5.0 pg/ml, $P < 0.04$ for IL-10). Regardless of steroid therapy or disease activity, serum levels of IFN- γ , TNF- α , IL-1 β , GM-CSF and IL-8 were no different in patients with sarcoidosis and controls. Vitamin D3 levels were significantly higher in patients with sarcoidosis *versus* normal controls (medians 78.0 *versus* 56.0, $P < 0.001$), active sarcoidosis ($n = 20$) *versus* inactive disease ($n = 17$) (medians 81.5 *versus* 66.0, $P < 0.03$) and active sarcoidosis *versus* controls (medians 81.5 *versus* 56.0, $P < 0.0002$). The levels were no different between patients with inactive sarcoidosis and controls. We suggest that IL-10 and vitamin D3 may contribute to the peripheral anergy in sarcoidosis. The elevated serum sCD23 suggests an increase in peripheral humoral immunity. Consistent with a quiescent peripheral immune system, factors capable of monocyte/macrophage activation (TNF- α , IFN- γ , GM-CSF and IL-8) were not elevated in the peripheral circulation.

Keywords sarcoidosis cytokines sCD23 vitamin D3

INTRODUCTION

Sarcoidosis manifests several different patterns of immune reactivity at different sites in the same individual [1,2]. In peripheral tissues hypergammaglobulinaemia and anergic skin test reactivity suggest enhanced B cell and deficient T cell function [2,3]. In contrast, activated T cells [4] and granulomas at the sites of active inflammation confirm exaggerated cell-mediated immunity (CMI) [1,2]. While many proinflammatory and Th1-type cytokines are increased in bronchoalveolar lavage fluid (BALF) [5,6], the situation regarding the peripheral circulation is unclear. Additionally, cytokines and other factors which may promote peripheral anergy have received little attention.

On the basis of previous research into immune dysregulation in sarcoidosis, we measured the levels of several factors important in different aspects of immune regulation in patients with well characterized sarcoidosis. Thus serum interferon-gamma (IFN- γ) was used as a measure of cellular immunity and IL-10 and vitamin D3 as anti-inflammatory agents [7,8]. Neutrophil activation was assessed by serum measurements of IL-8 and monocyte/macrophage function by serum measurements of IL-1 β , granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-alpha (TNF- α). Humoral immunity was assessed by measurement of serum soluble CD23 (sCD23), which is predominantly of B cell origin [9] and reflects activation and proliferation of this cell lineage [10,11]. We have also found sCD23 helpful in determining the overall balance of cellular and humoral immunity [12], owing to its stimulation by IL-4 and inhibition by IFN- γ [10,11].

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PATIENTS AND METHODS

Patients with sarcoidosis aged between 18 and 65 years were recruited from the disease register at the Prince Charles Hospital compiled by R.K.A. and from the out-patient clinics at this hospital. The diagnosis of sarcoidosis was based on the following: a clinical history compatible with sarcoidosis along with associated radiographic findings (bilateral hilar lymphadenopathy or interstitial lung disease), biochemical data (raised serum angiotensin-converting enzyme (ACE), hypercalcaemia), bronchoscopic features (increased CD4 T lymphocytes in BALF) and histological evaluation of mediastinal lymph nodes or transbronchial lung biopsy showing non-caseating granulomas. Patients who had suffered a viral or severe bacterial infection within the preceding 1 month were excluded from analysis. Each patient had clinical and laboratory data recorded on a special computer proforma and database, which allowed rapid evaluation of serial results [13]. Disease activity assessment utilized this information and was based on the observations of Baudouin & du Bois [14] and Thomas & Hunninghake [15]. In particular, disease activity was suggested by two or more of the following: (i) new or progressive symptoms/signs; dyspnoea, mononeuritis, skin lesions, sicca symptoms, fundal changes, uveitis, hepatomegaly/hepatosplenomegaly and lymphadenopathy; (ii) elevated serum calcium and ACE, raised 24 h urine calcium excretion and progressive hypergammaglobulinaemia; (iii) alterations in serial chest x-rays; new bilateral hilar lymphadenopathy, new or progressive pulmonary infiltrates; (iv) changing spirometry; worsening restrictive/obstructive lung function and decreasing transfer factor; (v) bronchoalveolar lavage showing increased lymphocytes; (vi) transbronchial biopsy showing non-caseating granulomas; (vii) gallium scan showing increased uptake in mediastinum, hilar glands, spleen or parotids.

The control group comprised healthy laboratory staff also free of infection within the preceding 1 month and of a similar age and sex distribution to the patients with sarcoidosis. Serum from patients and controls was separated and stored (-70°C) within 3 h of venesection and thawed immediately before use.

Soluble CD23 and cytokine measurements

Soluble CD23 was measured using a double monoclonal sandwich ELISA employing the EBVCS2 MoAb for capture and horseradish peroxidase (HRP)-conjugated BU38 for detection, as previously described [16]. Cytokines were measured using in-house ELISAs and commercially available paired MoAbs for IL-10, TNF- α and GM-CSF and recombinant cytokine standards as previously described [14]. IL-1 β was measured using a mouse anti-human IL-1 β MoAb capture and a polyclonal biotinylated rabbit anti-human IL-1 β detection (both from Cistron Biotechnology, Pine Brook, NJ). Recombinant IL-1 β for the standard curve was obtained from the National Institute for Biological Standards and Controls (Potters Bar, UK). Briefly, microtitre plates (Nunc Maxisorb, Melbourne, Australia) were coated overnight at 4°C with $100\ \mu\text{l}$ per well of $1\ \mu\text{g}/\text{ml}$ of appropriate MoAb in carbonate buffer. The plates then were washed six times in PBS and blocked with 10% newborn calf serum in PBS (NCS/PBS). The standard curve and duplicate $100\ \mu\text{l}$ of the neat test sera were then incubated for 3 h. After six washes in PBS, plates were then incubated for 90 min with $100\ \mu\text{l}$ per well of $1\ \mu\text{g}/\text{ml}$ of the biotinylated detection MoAb in NCS/PBS or the second stage rabbit anti-human polyclonal antibody. In the final stage plates were incubated with avidin-peroxidase (Sigma Aldrich Pty Ltd, Castle Hill, Australia)

$5\ \mu\text{g}/\text{ml}$ in NCS/PBS or a sheep anti-rabbit polyclonal antibody (Silenus Laboratory, Hawthorne, Australia) for 45 min and developed with *o*-phenylenediamine (OPD) substrate. There was negligible cross-reactivity between all the cytokines tested (IL-1 β , IL-8, IL-10, TNF- α , GM-CSF and IFN- γ) and with sCD23, IL-4 and IL-6. IFN- γ levels were measured using a commercial ELISA (CSL, Melbourne, Australia) with a detection limit of $20\ \text{pg}/\text{ml}$. The detection limit for IL-10 and TNF- α was $5\ \text{pg}/\text{ml}$, for IL-1 β it was $10\ \text{pg}/\text{ml}$ and for GM-CSF it was $2\ \text{pg}/\text{ml}$. In all cases patient and control sera were randomly scattered on each plate.

Vitamin D3 measurement

This was based on a 1,25-dihydroxyvitamin D-specific thymus receptor incorporating preliminary extraction and purification of vitamin D metabolites using a $\text{C}_{18\text{OH}}$ cartridge and a non-equilibrium competitive protein binding assay (Incstar Corp., Stillwater, MN).

Statistical analysis

The Mann-Whitney and Pearson's correlation tests were used throughout.

RESULTS

All 37 patients with sarcoidosis were Caucasian and from the surrounding community (M:F 22:15; mean age 44 years). Thirteen were on steroid immunosuppression for presumed active sarcoidosis (M:F 8:5; mean age 40 years) and 24 were not on this therapy. Of the latter, seven with active disease had never received steroids (M:F 4:3; mean age 48 years) and 17 with inactive disease had either never received steroids or had finished them a minimum of 3 months previously (M:F 10:7; mean age 45 years). There was no significant difference in the male:female ratio or mean age between patients with sarcoidosis and control subjects (M:F 11:9; mean age 38 years). Among the patients with sarcoidosis, seven patients had a completely normal chest x-ray, 13 had bilateral hilar lymphadenopathy (BHL), 10 had BHL and pulmonary fibrosis and seven had only pulmonary fibrosis. All patients with sarcoidosis on steroids had been on steroid immunosuppression for at least 3 months and on the same dose of prednisone for at least 1 month.

Patients with active sarcoidosis not on steroids had a significantly higher level of serum ACE than those with inactive disease not on steroids (medians 241 *versus* 152, $P < 0.005$) (normal range 30–170 U/l) and those with probably active disease on steroids (median 241 *versus* 144, $P < 0.002$). There was no significant difference in the level of serum ACE between patients with inactive sarcoidosis not on steroids and control subjects (152 *versus* 128, $P > 0.2$). Serum ACE was no different between patients with normal chest x-ray, BHL with and without fibrosis and fibrosis only.

Serum levels of sCD23 and IL-10 were significantly elevated in patients with sarcoidosis relative to control subjects (median 13.9 *versus* 9.5 arbitrary units/ml, $P < 0.01$ for sCD23 and 9.6 *versus* 5.0 pg/ml , $P < 0.04$ for IL-10) (Figs 1 and 2, respectively). As we have previously found steroid immunosuppression to depress levels of serum sCD23 [17], we separated patients with sarcoidosis into those receiving and not receiving steroids. As expected, patients not on steroids had a higher level of serum sCD23 than those on steroids. While this result was not statistically different, a comparison of the sarcoidosis group not on steroids with control subjects did show a highly significant difference in serum sCD23 (median 15.2 *versus* 9.5, $P < 0.001$) (Fig. 1). For both serum sCD23

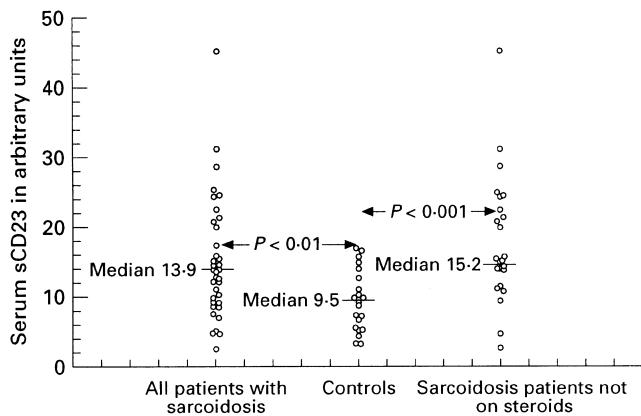


Fig. 1. Scatterplot of serum sCD23 (in arbitrary units/ml) in patients with sarcoidosis and normal controls.

and IL-10 there was, however, no significant difference between patients with active and inactive disease. Serum levels of IL-10 were not significantly different between patients receiving and not receiving steroids, and serum sCD23 showed no correlation with serum IL-10.

Regardless of steroid therapy or disease activity, serum levels of IFN- γ , TNF- α , GM-CSF and IL-8 were no different in patients with sarcoidosis and controls (data not shown). Additionally, serum cytokines and sCD23 were no different between patients with normal chest x-ray, BHL with and without fibrosis and fibrosis only. The levels of serum vitamin D3 were significantly higher in patients with sarcoidosis relative to controls (median 78.0 versus 56.0, $P < 0.001$) (Fig. 3), active sarcoidosis whether on steroid treatment or not ($n = 20$) versus inactive disease ($n = 17$) (median 81.5 versus 66.0, $P < 0.005$) and active sarcoidosis whether or not on steroid treatment versus controls (median 81.5 versus 56.0, $P < 0.0002$). There was no significant difference in vitamin D3 between patients with inactive sarcoidosis and controls (median 66.0 versus 56.0, $P = 0.1$) (Fig. 3) and between patients with active sarcoidosis on steroid treatment and those not on steroids (median 83 versus 74, $P > 0.6$).

In both patients and control subjects, there was a significant positive correlation between serum levels of vitamin D3 and IL-10 ($r = 0.381$, $P < 0.003$) (Fig. 4). Between IFN- γ and vitamin D3 a significant negative correlation was evident ($r = -0.285$, $P < 0.03$) (Fig. 5). There was no significant correlation between serum levels

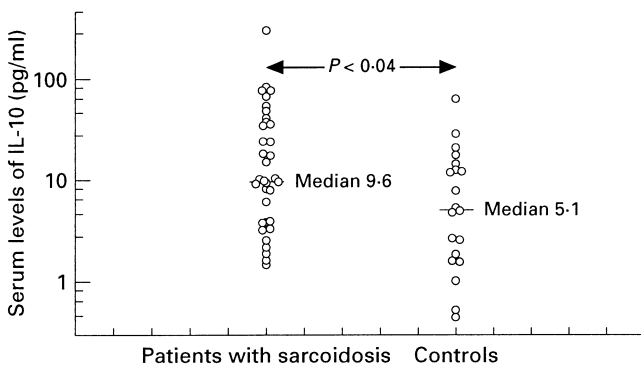


Fig. 2. Scatterplot of serum IL-10 (in pg/ml) in patients with sarcoidosis and normal controls.

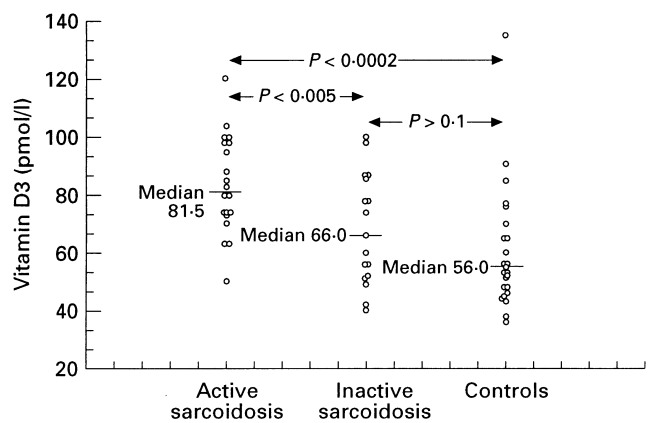


Fig. 3. Scatterplot of vitamin D3 (in pmol/l) in patients with sarcoidosis and normal controls.

of sCD23 and serum levels of IFN- γ , GM-CSF and vitamin D3. There were no other significant correlations between IFN- γ , IL-10, GM-CSF and vitamin D3.

DISCUSSION

The assessment of disease activity in sarcoidosis is difficult. Coupled with differences in assay techniques and the tissue or body compartments analysed it is not surprising that there are many inconsistencies in the literature on the precise pattern of immune dysfunction in sarcoidosis. Nevertheless, two patterns of immune activity are evident in sarcoidosis. At the sites of disease activity, granulomatous inflammation is highly suggestive of exaggerated cellular immunity. Thus previous reports have shown increased expression of mRNA for IFN- γ inside granulomas [18,19] and the frequent secretion of both IFN- γ and IL-2 by BALF-derived T cell clones [20]. Peripherally, however, depressed cellular immunity is evident. In the skin this is manifest as cutaneous anergy to commonly encountered antigens and commonly attributed to the CD4 T lymphopenia frequently observed in sarcoidosis. In the blood it is suggested by the infrequent production of IFN- γ by peripheral blood-derived T cell clones and by the fact that peripheral blood mononuclear cells (PBMC) manifest significantly depressed IFN- γ production compared with normal

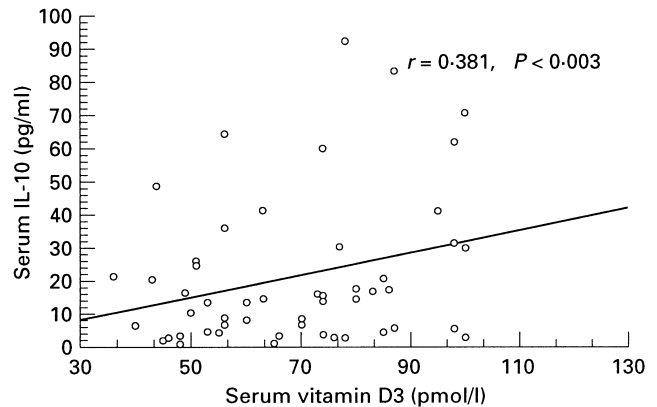


Fig. 4. Variation between serum vitamin D3 and IL-10 in patients with sarcoidosis.

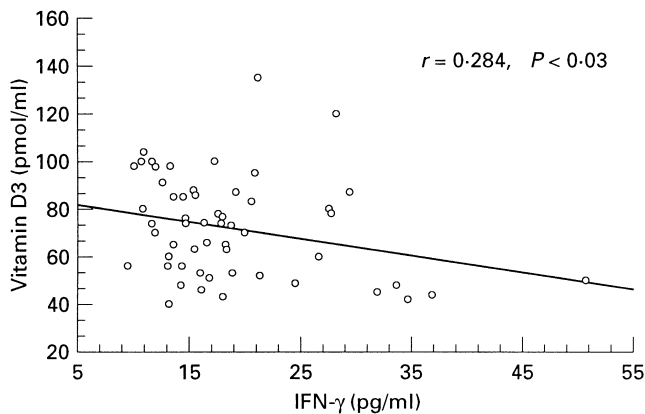


Fig. 5. Variation between serum vitamin D3 and IFN- γ in patients with sarcoidosis.

controls [19]. However, serum levels of IFN- γ have been found to be elevated in patients with sarcoidosis [21,22], and particularly so in patients with active disease. We found no such difference in our patients, a finding which may be related to the small numbers of patients with active disease not on steroids and the general difficulty in assessing disease activity. This may also have contributed to the absence of any difference in serum sCD23 and IL-10 between patients with active and inactive disease when these proteins were increased in the sarcoidosis group as a whole.

The finding of raised levels of serum IFN- γ in patients with sarcoidosis [21,22] would appear to be at odds with the depressed peripheral CMI and hypergammaglobulinaemia observed in this condition. As such it would necessitate a down-regulation of IFN- γ receptors on PBMC or the circulation of antigenically intact but functionally impaired IFN- γ . Alternatively, the peripheral anergy in sarcoidosis may be exclusively the result of non-specific CD4 T cell sequestration within the sites of disease activity. However, peripheral blood T cells from patients with sarcoidosis have been reported to show normal production of IFN- γ when stimulated with a combination of phytohaemagglutinin (PHA) and supernatants from PHA-stimulated macrophages from normal subjects [23]. This response was abrogated by incubation with the supernatants from PHA-stimulated macrophages from patients with sarcoidosis. Indeed, the response of T cells from normal subjects incubated with sarcoid macrophages or supernatants from PHA-stimulated sarcoid macrophages was also depressed [23]. Thus, activated macrophages from patients with sarcoidosis may produce a factor which can depress the activation and production of IFN- γ by peripheral blood T cells. While the identity of this circulating factor is unclear, possibilities would include an antagonist of T cell IFN- γ production such as a Th2 cytokine or an agent capable of inhibiting lymphocyte cytokine production and proliferation such as vitamin D3 [8].

Hoshino *et al.* [20] have recently assessed cytokine production by peripheral blood and BALF-derived T cell clones from patients with sarcoidosis. They observed a significantly increased production of IL-10 by both types of T cell clones when compared with peripheral blood T cell clones derived from healthy controls. However, peripheral blood T cell clones from patients with sarcoidosis produced similar, if not less IFN- γ than those from healthy controls. In keeping with these findings, our results confirm essentially normal levels of serum IFN- γ and significantly elevated

levels of IL-10 in patients with sarcoidosis relative to normal controls. This skewing of the peripheral immune system towards relatively higher humoral immunity is confirmed by our separate finding of significantly elevated levels of soluble CD23 in patients with sarcoidosis; serum levels of sCD23 being maintained by the opposing actions of stimulatory IL-4 and inhibitory IFN- γ [10,11]. Soluble CD23 promotes the survival of centrocytes within the germinal centres of lymph nodes by the activation of the *bcl-2* proto-oncogene [24]. Thus the elevated serum levels suggest an explanation for the hypergammaglobulinaemia frequently observed in sarcoidosis. Of interest, the combination of elevated serum sCD23 and IL-10 is very similar to that observed in patients with rheumatoid arthritis [25,26]. As in sarcoidosis, this manifests exaggerated peripheral humoral immunity and increased cellular immunity within disease-affected tissues.

The quiescent state of the peripheral immune system in sarcoidosis is confirmed by the cutaneous anergy which is a recognized feature of sarcoidosis. In addition, previous reports have confirmed essentially normal serum levels of the proinflammatory cytokines TNF- α and IL-1 β [19]. This contrasts with the increased levels of these cytokines in lymph node extracts [19] and the increased expression of IL-1 β mRNA in sarcoid lymph nodes reported by Devergne *et al.* [18]. Furthermore, Hoshino *et al.* [20] have reported markedly increased production of TNF- α , IL-1 α and IL-6 by T cell clones derived from BALF compared with the peripheral blood. More recently, Bäumer *et al.* [27] observed essentially normal numbers of Th1-type IFN- γ -secreting clones from the peripheral blood of nine patients with sarcoidosis. Our results confirm the quiescent state of the peripheral immune system in sarcoidosis, in that we observed no statistical difference in levels of serum IL-1 β and TNF- α in patients with sarcoidosis and normal controls. Furthermore, we found no increase in those cytokines capable of peripheral monocyte and macrophage activation (IL-8, IFN- γ and GM-CSF). Even using potent non-specific mitogenic stimuli it has proved difficult to overcome the peripheral anergy of sarcoidosis [21,23]. Indeed peripheral cutaneous reactivity is frequently maintained only to the Kveim-Siltzbach (K-S) antigen and to autoclaved autoinoculated BALF macrophages from patients with sarcoidosis [28]. Moreover, BALF cells and lung biopsy tissue from patients with sarcoidosis show increased expression of IL-1 β and IFN- γ mRNA [29] as well as heightened spontaneous and stimulated cytokine production [29–31]. Assessment of the Th1/Th2 cytokine profile of K-S antigen-stimulated PBMC and BAL cells may provide insight into the importance and relevance of this concept in sarcoidosis.

The compartmentalization of immune dysfunction in sarcoidosis suggests a possible circulating immune suppressive factor responsible for the peripheral anergy. Our findings of elevated serum IL-10 and vitamin D3 in patients with sarcoidosis suggests that these may be two such immune suppressive factors. As such, there was a significant positive correlation between IL-10 and vitamin D3 (Fig. 4, $P < 0.003$). IL-10 is a potent anti-inflammatory cytokine capable of suppressing MHC class I and II expression by macrophages and other antigen-presenting cells [7]. The source of the elevated IL-10 is unclear. It is, however, likely to be of non-pulmonary origin, as the concentration of IL-10 in the BALF of patients with sarcoidosis was recently reported to be significantly below that of healthy controls [6]. Regarding vitamin D3, this has been reported to inhibit lymphocyte proliferation and the production of IFN- γ , IL-2, IL-1 α , IL-6 and TNF- α [8]. In keeping with this, we observed a significant negative correlation between serum

vitamin D3 and IFN- γ (Fig. 5). The production of vitamin D3 by pulmonary alveolar macrophages (PAM) is, however, encouraged by IFN- γ via a stimulation of the 25OH D3-1-hydroxylation reaction. This occurs via the intermediacy of five lipoxygenase arachidonate metabolites and is inhibited by the lipoxygenase inhibitor BW755C [32]. Thus the heightened spontaneous production of IFN- γ by unstimulated and concanavalin A (Con A)-stimulated PBMC [33] may be explained by the removal of vitamin D3 and IL-10 during the PBMC separation procedure. As the mRNA levels of IFN- γ have recently been reported to be increased in the PBMC of stage I sarcoidosis [34], it is likely that vitamin D3 may act to prevent mRNA translation. Thus the elevated levels of sCD23, vitamin D3 and IL-10 we have found in patients with sarcoidosis are entirely consistent with the normal levels of IFN- γ and TNF- α observed in these patients.

In conclusion, our results confirm significantly raised levels of serum vitamin D3 and IL-10 in patients with sarcoidosis. Along with the peripheral blood CD4 T lymphopenia observed, they suggest another possible cause for the peripheral anergy in this condition. A relative increase in peripheral humoral immunity was suggested by the elevated level of serum sCD23. This provides a possible explanation for the hypergammaglobulinaemia frequently found in sarcoidosis. Circulating factors (TNF- α , IFN- γ , GM-CSF and IL-8) capable of monocyte and macrophage activation were not increased.

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